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Dysregulation of the glutamine transporter Slc38a3 (SNAT3) and ammoniagenic enzymes in obese, glucose-intolerant mice

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Abstract: BACKGROUND/AIMS Uric acid nephrolithiasis is prevalent among patients with type 2 diabetes and metabolic syndrome; it is correlated with an acidic urine and lower urinary ammonium excretion and is likely associated with insulin resistance. Insulin stimulates ammoniagenesis in renal cell lines via increased phosphate-dependent glutaminase (PDG) activity and glutamine metabolism. Ammonium excretion into the proximal tubule is mediated at least in part by the Na(+)/H(+)-exchanger NHE3 and in the collecting duct involving the Rhesus protein RhCG. Here we tested, whether obesity and insulin resistance in a diet-induced mouse model could contribute to deranged ammonium excretion. **METHODS** Obesity was induced by diet in mice and the impact on key molecules of proximal tubular ammoniagenesis and urinary acid excretion tested. **RESULTS** Diet-induced obesity was confirmed by pathological intraperitoneal glucose tolerance tests (IPGTT). Three groups of mice were compared: control mice; obese, glucose-intolerant with abnormal IPGTT (O-GI); or moderate weight with normal IPGTT (Non-Responders, NR). Basal urinary ammonium excretion did not differ among groups. However, acid loading increased urinary ammonium excretion in all groups, but to a lesser extent in the O-GI group. SNAT3 mRNA expression was enhanced in both obese groups. PDG expression was elevated only in acid-loaded O-GI mice, whereas PEPCK was enhanced in both O-GI and NR groups given NH₄Cl. NHE activity in the brush border membrane of the proximal tubule was strongly reduced in the O-GI group whereas RhCG expression was similar. **CONCLUSION** In sum, obesity and glucose intolerance impairs renal ammonium excretion in response to NH₄Cl feeding most likely through reduced NHE activity. The stimulation of SNAT3 and ammoniagenic enzyme expression may be compensatory but futile.

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Dysregulation of the glutamine transporter Slc38a3 (SNAT3) and ammoniagenic enzymes in obese, glucose-intolerant mice

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ABSTRACT

Uric acid nephrolithiasis is prevalent among patients with type 2 diabetes and metabolic syndrome; it is correlated with an acidic urine and lower urinary ammonium excretion and is likely associated with insulin resistance. Insulin stimulates ammoniogenesis in renal cell lines via increased phosphate-dependent glutaminase (PDG) activity and glutamine metabolism. Ammonium excretion into the proximal tubule is mediated at least in part by the Na^+/H^+ -exchanger NHE3 and in the collecting duct involving the Rhesus protein RhCG. Here we tested, whether obesity and insulin resistance in a diet-induced mouse model could contribute to deranged ammonium excretion. Diet-induced obesity was confirmed by pathological intraperitoneal glucose tolerance tests (IPGTT). Three groups of mice were compared: control mice; obese, glucose-intolerant with abnormal IPGTT (O-GI); or moderate weight with normal IPGTT (Non-Responders, NR). Basal urinary ammonium excretion did not differ among groups. However, acid loading increased urinary ammonium excretion in all groups, but to a lesser extent in the O-GI group. SNAT3 mRNA expression was enhanced in both obese groups. PDG expression was elevated only in acid-loaded O-GI mice, whereas PEPCK was enhanced in both O-GI and NR groups given NH_4Cl . NHE activity in the brush border membrane of the proximal tubule was strongly reduced in the O-GI group whereas RhCG expression was similar. In sum, obesity and glucose intolerance impairs renal ammonium excretion in response to NH_4Cl feeding most likely through reduced NHE activity. The stimulation of SNAT3 and ammoniagenic enzyme expression may be compensatory but futile.

INTRODUCTION

The prevalence of diabetes from a global perspective has reached approximately 170 million and is expected to rise further [1]. Interestingly, the incidence of nephrolithiasis in this population has increased in parallel, and it has been suggested that a relationship may exist between these metabolic disorders and renal stone formation [2-4]. A recent cross-sectional analysis comparing three large epidemiological studies over a 44 year period found that diabetes appeared to be a significant risk factor for developing uric acid nephrolithiasis (UAN), and had suggested that insulin resistance could be the effector [5]. UAN is highly associated with low urinary pH and urine volume, hyper- or normouricosuria, and the increased incidence of diabetes or insulin resistance [6-10]. Pharmacological treatment with potassium or sodium citrate has been proven to increase urinary pH and inhibit UA stone formation, providing further evidence that UAN is linked to low urinary pH [11, 12] [13, 14]. Patients with diabetes and UAN not only have low urinary pH values, but also exhibit decreased renal ammonium excretion. The mechanism likely involves defective renal ammoniogenesis or excretion since a low urinary pH usually is associated with enhanced ammonium excretion in the urine [7, 11, 15-18]. Furthermore, functional acid-loading tests in these patients resulted in a reduced capacity to acidify their urine in comparison to healthy controls [7].

Renal ammoniogenesis and gluconeogenesis increase substantially in the proximal tubule (PT) in response to acute or chronic metabolic acidosis. This is a direct result of the shunting of glutamine from the visceral or splanchnic pool to the kidney, with concomitant increases in glutamine extraction from blood [19, 20]. We and others have shown that glutamine influx is likely due to the basolateral proximal tubule glutamine transporter Slc38a3 (SNAT3), since increased SNAT3 expression has been observed during metabolic acidosis [21-25]. SNAT3 (Slc38a3) mediates the transport of glutamine while Na^+ and H^+ ions are exchanged [26]. Recent work by our group has also shown enhanced SNAT3 mRNA and protein in response to potassium restriction or a high protein diet, conditions that create an acidosis-like state and stimulate renal ammoniogenesis [27-34]. Glutamine is then transported into the mitochondria

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of the PT cell and metabolized. One ammonium ion is liberated through the deamidation of glutamine to glutamate via phosphate-dependent glutaminase (PDG), while another ammonium ion is produced upon conversion of glutamate to α -ketoglutarate. In the PT cell cytosol, α -ketoglutarate has two metabolic fates; as a substrate in the tricarboxylic acid cycle, or as a precursor of *de novo* glucose synthesis in the presence of phosphoenolpyruvate carboxykinase (PEPCK), resulting in the production of two bicarbonate molecules. Increases in PDG and PEPCK enzyme activity during metabolic acidosis have been associated with a concomitant increase in mRNA levels and protein abundance and are thus important regulators of ammoniogenesis [35-39]. These two enzymes are, however, differentially regulated. PDG is augmented during MA as a result of increased stabilization of its mRNA secondary to the binding of ξ -crystallin/NADPH:quinone reductase to a pH responsive AU-rich region in the 3' untranslated region of its mRNA, while increases in PEPCK are mostly the result of augmented transcription of the PEPCK gene [39].

Once ammonium is produced in the PT, it is transported into the tubular lumen. This is accomplished either via diffusion as NH_3 where it can trap protons, or it can be transported as ammonium. A key protein involved in the luminal transport of ammonium as well as providing protons for bicarbonate reabsorption is the Na^+/H^+ exchanger, NHE3, localized to the brush border membrane of the PT and increased during NH_4Cl -induced MA [40-42]. Reabsorption of ammonium produced by the PT occurs in the medullary thick ascending limb largely by the activation of a luminal $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporter (NKCC2) [43]. After accumulation in the medullary interstitium, a process dependent on sulfatides, ammonium is ultimately excreted in the final urine, via the Rhesus factor Rhcg protein [44-47].

The ammoniagenic pathway is also regulated by several hormones: angiotensin II and insulin stimulate ammoniogenesis whereas prostaglandin PGF_2 reduces it [48-50]. The renal proximal tubule is also an important gluconeogenic site and in contrast to ammoniogenesis gluconeogenesis is reduced by insulin [51-55]. Induction of diabetes in rats *in vivo* or in renal cortex slices results in increased gluconeogenesis, while supplying insulin results in a

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substantial decrease in gluconeogenic activity [56-58]. Further, insulin can also stimulate NHE3 activity in the proximal tubule [59-62]. A recent study has shown that Zucker diabetic fatty rats with a mutation in the leptin receptor gene exhibit reduced urinary ammonium and pH, with a concomitant reduction in renal brush border membrane NHE3 activity, suggesting that renal ammonium excretion is reduced in part by a reduction in NHE3 activity [63].

Given that both ammoniagenesis and gluconeogenesis are closely linked in the renal proximal tubule cell, we hypothesized that a diet-induced, glucose-intolerant state could alter renal ammoniagenesis through dysregulation of the glutamine transporter SNAT3, renal ammoniagenic and gluconeogenic enzymes, PDG and PEPCK, or transport proteins involved in renal ammonium excretion. Modifications in the regulation of these proteins could ultimately result in perturbations in ammoniagenesis and excretion, and could help to explain the pathogenesis of UAN in subjects with diabetes and metabolic syndrome.

Animals

Male C57BL6 mice (n=72) were randomly assigned to two different dietary treatment groups and placed on either a standard diet (control, n = 24) or “cafeteria” style diet (n = 48) for 10 weeks. A cafeteria style diet consists of various combinations of energy dense foodstuffs, and is widely used as a model for diet-induced obesity [64]. Our experimental cafeteria diet consisted of unsalted ground peanuts (40%), cookies (25%) and chocolate (15%) mixed with standard rodent chow (20%) to ensure adequate macro- and micronutrient composition. The nutrient composition of the cafeteria diet was as follows: 460 kcal, 15 g of protein, 25 g of carbohydrate and 34 g of fat per 100 g of ground food. Mice had *ad libitum* access to food and water during the 10 week dietary treatment period, and were housed in climate controlled and 12 h light-cycled rooms. All mice were weighed weekly. Animal experiments were conducted according to the Swiss animal welfare laws and approved by the Swiss local animal authority, Zurich, Switzerland.

Intraperitoneal glucose tolerance tests (IPGTT)

After 10 weeks of dietary treatment, each mouse was subjected to IPGTT. The test was performed over a 2 h period. Mice were fasted overnight with free access to water. A single dose of glucose (2 mg/g body weight) was given upon initiation of testing, followed by blood collections from the tail vein every 30 minutes. Glucose monitoring was performed using the Accu-chek blood glucose meter (Roche). After the IPGTT, mice were divided into various groups depending on the test outcome: control with normal IPGTT; **Obese, Glucose-Intolerant** mice with abnormal IPGTT (subsequently named: O-GI); or moderately obese with relatively normal IPGTT (**Non-Responders**, NR). All mice were allowed to recover from the IPGTT for one week, and were subsequently housed in metabolic cages for the next series of experiments.

Metabolic cage studies

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Mice were individually placed in metabolic cages for a total of 4 days, and continued on the experimental diets. Control, O-GI and NR mice were subdivided into NH_4Cl treated groups; NH_4Cl was powdered into the food (2 g/100 g chow) for the final 48 h. Food and water intake, body weight and urinary output were monitored daily. During the final 48 h of dietary treatment, urine was collected every 24 hours under mineral oil and urinary pH was immediately assessed using a pH microelectrode (691 pH meter, Metroholm). The Berthelot and Jaffe methods were used to assess urinary $\text{NH}_3/\text{NH}_4^+$ and creatinine concentration [65, 66]. At the end of the experimental treatment, mice were anesthetized by intraperitoneal injection of ketamine and xylazine, and blood was collected in heparinized tubes, immediately centrifuged to extract serum, and frozen at -80°C . Mice were not perfused prior to harvesting kidneys given that neither SNAT3, nor PDG and PEPCK are expressed in whole blood or erythrocytes, respectively [67]. Although there is some evidence suggesting white blood cells exhibit PDG activity, exsanguination caused by the blood collections minimized the likelihood of blood cell contamination in our preparations [68]. Kidneys were harvested, flash-frozen in liquid nitrogen, and transferred to a -80°C freezer until further use.

Semi-quantitative Real-time RT PCR

Previously frozen kidneys were homogenized using a Rotor-stator homogenizer, and RNA was immediately extracted using Qiagen RNeasy Mini Kit (Qiagen; Hilden, Germany). DNase digestion was performed using the RNase-free DNase Set (Qiagen; Hilden, Germany). Total RNA extractions were analyzed using the NanoDrop ND-1000 spectrophotometer (Wilmington, DE, USA). cDNA was prepared from diluted RNA samples (100 ng/ μl) using the TaqMan Reverse Transcriptase Reagent Kit containing 10X RT buffer, MgCl_2 , random hexamers, dNTPs, RNase inhibitors and Multiscribe reverse transcription enzyme (Applied Biosystems/Roche; Foster City, CA, USA). Thermocycling conditions for reverse transcription were set at 25°C for 10 min, 48°C for 30 min, and 95°C for 5 min (Biometra TGradient thermocycler, Goettingen, Germany). Semi-quantitative Real-time RT-PCR was used to

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determine relative mRNA expression (7500 Fast Real-Time PCR system, Applied Biosystems).

Thermocycling conditions were set at: 50 °C (2 min), 95°C (10 min), 95 °C (15 sec; 40 cycles) and 60 °C (1 min). Forward and reverse primer concentration was 25 µM; probe concentration was 5 µM. TaqMan Universal PCR master mix 2X (Applied Biosystems/Roche) was used as the Taq polymerase. Primers and probes for SNAT3, phosphate dependent glutaminase (PDG), phosphoenolpyruvate carboxykinase (PEPCK), rhesus glycoprotein (Rhcg) and Hypoxanthine-guanine phosphoribosyltransferase (HPRT) were generated using the Primer Express software from Applied Biosystems and synthesized at Microsynth (Balgach, Switzerland) as described previously [25]. The primer and probe sequences for Rhcg were 5'-GTT GGA GAA GAA GCG CAA GAA-3', forward; 5'-CGA AGA CCA TGG CGT GTA CA-3', reverse; 5'-TTA CTA TCG CTA CCC GAG CTT CCA G-3', probe. Probes were generated with the reporter dye FAM at the 5' end and TAMRA at the 3' end. Each sample was run in triplicate including a negative control (without Multiscribe reverse transcription enzyme). The cycle threshold (C_t) values obtained were ultimately compared to C_t values of the endogenous gene HPRT. Relative mRNA expression ratios were calculated as $R=2^{[C_t(HPRT)-C_t(\text{gene of interest})]}$.

Immunoblotting

Total membrane and cytosolic protein preparations were prepared from whole, non-perfused kidneys using a K-HEPES buffer composed of 200 mM mannitol, 80 mM HEPES, 41 mM KOH, along with the protease inhibitors, PMSF, K-EDTA, and leupeptin (pH 7.5). Kidneys were homogenized in 200 µl of ice-cold K-HEPES buffer using a tip sonicator and immediately centrifuged at 2000 rpm for 20 min (4°C). The supernatant was aspirated and placed in an ultracentrifuge (Sorvall, Thermo Fischer Scientific) for 1 h at 41,000 rpm 4°C. The supernatant containing cytosolic protein from this second centrifugation step was removed and the pellet containing membrane protein was resuspended in K-HEPES buffer and sonicated to evenly distribute proteins. The BioRad Dc Protein assay was used to measure protein concentration (Bio-Rad; Hercules, CA, USA). Total membrane (75 µg) or cytosolic (50 µg) protein containing

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Laemmli sample buffer and loaded onto a 10% polyacrylamide gel and SDS-PAGE was performed. Proteins were transferred to polyvinylidene difluoride membranes (PVDF; Immobilon-P, Millipore, Bedford, MA, USA). Membranes were blocked with Tris-buffered saline / 0.1% Tween and 5% non-fat dry milk for 1 h. Primary antibodies were applied for 2 h at RT or overnight at 4°C. The primary antibodies used for this study were: phosphate-dependent glutaminase (PDG), which recognizes both the rat (KGA) and human (GAC) kidney-type isoforms of PDG forming the mature PDG protein (66 and 68 kDa; a kind gift from N. Curthoys, Colorado State University, USA; diluted 1:500) [69]; anti-PEPCK polyclonal antibody (63 kDa; Cayman Chemical, Ann Arbor, MI, USA; diluted 1:1,000), and mouse monoclonal antibody against β -actin (42 kDa; Sigma, St. Louis, MO, USA; diluted 1:5,000). The anti-Slc38a3 antibody (1:500) was generated as previously described [24]. The secondary antibodies used were anti-rabbit alkaline phosphatase conjugated diluted 1:5,000, and anti-mouse IgG alkaline phosphatase conjugated diluted 1:5,000 or anti-mouse IgG horseradish peroxidase-conjugated 1:10,000, and donkey anti-rabbit HRP 1:12,000. After a series of washing steps and blocking, membranes were treated with alkaline phosphatase- or horseradish peroxidase-conjugated developing solution (Immobilon, location) and exposed to the Diana III chemiluminescence detection system (Raytest). Specific bands on PVDF membranes were later quantified using AIDA Image analyzer version 3.44. Membranes were stripped and reprobed for anti- β -actin and subsequent analysis of the protein of interest was determined relative to β -actin quantification and reported as relative protein abundance (ratio of β -actin/protein of interest).

Brush border membrane acridine orange quenching experiments

Renal brush border membrane vesicle preparations (BBMV) were prepared as previously described [70]. Briefly, kidneys were cut into small sections and homogenized using a Polytron homogenizer with a fine rod for 2 minutes in a buffer containing: 300 mM mannitol, 5 mM EGTA and 12 mM Tris-HCl, pH 7.1. A fraction of the homogenates was stored at -20 °C for

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immunoblotting, while 1 M MgCl_2 was added to the remaining homogenate and allowed to precipitate on ice for 15 minutes. The sample was then centrifuged at 4500 rpm for 15 minutes. The supernatant was aspirated and centrifuged at 18000 rpm for 30 minutes. The pellet was resuspended in membrane buffer consisting of: 300 mM mannitol, 20 mM HEPES-Tris, pH 7.4. Samples were then centrifuged at 18500 rpm for 30 minutes. Vesicle buffer was added to samples, containing: 280 mM mannitol, 5 mM MES/*N*-methyl-D-glutamine and 2 mM MgCl_2 , pH 5.5. Protein concentration was measured using the BioRad fast method.

Acridine orange quenching measurements were performed as previously described [71]. Measurements were performed in a Shimadzu RF-5000 spectrofluorometer equipped with a thermostated cuvette (kept at 25°C). BBMV were dissolved in a buffer containing 280 mM mannitol, 5 mM Mes, and 2 mM MgCl_2 (adjusted to pH 5.5 with *N*-methyl-d-glucamine). Acridine orange was excited at 493 nm, and emission was monitored at 530 nm. The cuvette was filled with 2 ml of buffer (240 mM mannitol/20 mM Hepes/2 mM MgCl_2 , adjusted to pH 7.5 with *N*-methyl-d-glucamine), containing 6 μM acridine orange. The experiment was started by injecting 30 μl of BBMV suspension. After 60 s of equilibration, NHE activity was initiated by injection of 80 μl of 2 M Na gluconate. NHE activity was calculated as ratio of ΔpH per min over Q , where Q is the initial quenching after injection of BBMV. All experiments were done at least in quadruplicates.

Statistical analyses were performed using unpaired Student's *t*-test, and results with $p < 0.05$ were considered statistically significant. Data are reported as means \pm SEM.

“Cafeteria-diet” feeding induces obesity and pathological glucose intolerance in mice

After 10 weeks of a high fat, high energy “cafeteria-style diet”, or a standard control diet, all mice (n=72) were subjected to intraperitoneal glucose tolerance testing (IPGTT). Control mice had the lowest body weight (31.3 ± 0.6 g) and showed no evidence for glucose intolerance (figure 1A, table 1). IPGTT revealed two distinct tiers of glucose intolerance among the cafeteria-fed mice. Approximately half of the cafeteria-fed mice were significantly obese (43.5 ± 0.9 g) and did not reach euglycemia at the end of the IPGTT (**O**bese, **G**lucose-**I**ntolerant; O-GI group), while the other half of cafeteria-fed mice were moderately obese (37.8 ± 1.4 g) and clearly less glucose intolerant (**N**on-**R**esponders; NR group). A similar phenomenon has been described in rats showing that chronic consumption of a high fat, high calorie diet led to a weight differential in roughly half of the study population tested, resulting in obesity and insulin resistance [72-74]. Furthermore, these observations were noted without a further increase in food intake in these animals, which has been suggested to be associated with irreversible changes in body weight and adiposity set-points.

Abnormal urinary acid excretion in obese, glucose-intolerant mice

Mice from all three groups (n = 48) were placed in metabolic cages after one week of recovery from IPGTT and maintained on their respective diets. The O-GI, NR and control groups were subdivided and challenged with an acid load consisting of NH_4Cl powdered into the food for 48 h (2 g/100 g food). Food intake in the control groups was highest in the lean mice and similar between the O-GI and NR groups (control: 3.55 ± 0.16 g /day; O-GI: 2.02 ± 0.09 g/day; NR: 2.13 ± 0.11 g/day). NH_4Cl addition reduced daily food intake in all groups but remained higher in the lean mice and was not significantly different between the two obese groups (control: 2.71 ± 0.21 g /day; O-GI: 1.27 ± 0.13 g/day; NR: 1.65 ± 0.08 g/day). Thus, NH_4Cl intake

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was higher in the lean group but comparable in the two obese groups. The similar intake of NH_4Cl in the obese groups is also reflected by similar rates of urinary chloride excretion in these two groups. Moreover, dietary acid load from involatile acids was also similar between these two groups as indicated by similar rates of urinary sulfate and phosphate excretion (table 1).

At baseline, there were no differences in urinary pH or ammonium excretion between groups. Differences were noted after the acid load was provided. Acid-loading resulted in significantly lower urinary pH in all groups in comparison to respective controls (Figure 2A, table 1). Although most clinical data show an acidic urine pH in patients with metabolic syndrome [6, 7, 9], we observed a more alkaline urinary pH in O-GI/ NH_4Cl treated mice than acid-loaded control mice fed a standard diet. Urinary electrolytes were analyzed to confirm the effect of acid-loading (table 1). Urinary phosphate levels were unremarkable between groups, however 24 h phosphate excretion relative to urine volume showed reduced phosphate excretion in the O-GI group compared to controls (table 1). All acid-challenged mice had increased urinary ammonium excretion as expected, however, O-GI mice given NH_4Cl had less ammonium excretion relative to urine volume (figure 2B, table 1).

Dysregulation of SNAT3 and key ammoniagenic enzymes in obese, glucose-intolerant mice

We and others have previously shown that gene expression of SNAT3, and the key ammoniagenic enzymes PDG and PEPCK increases after inducing acidosis with NH_4Cl treatment [21, 22, 24, 25, 28, 37, 39, 75]. All acid-loaded groups showed enhanced SNAT3 mRNA and protein levels (figure 3A-K), however each group showed varying expression. O-GI mice treated with NH_4Cl had 2-fold higher SNAT3 mRNA abundance in comparison to controls given NH_4Cl (figure 3H). SNAT3 protein abundance was also 1.4-fold higher in O-GI/ NH_4Cl treated mice in comparison to NR/ NH_4Cl treated mice (figure 3J) despite lower total NH_4^+ excretion. Similarly, immunoblotting revealed elevated SNAT3 protein abundance in O-GI/ NH_4Cl mice when compared to acid-loaded control mice (figure 3, H and I). PDG expression followed similar trends, with increased mRNA expression in all groups treated with NH_4Cl (figure 4A). Acid-

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challenged obese mice (both O-GI and NR) showed relatively more PDG protein abundance than respective controls, similar to mRNA expression (figure 4, A-E). O-GI/NH₄Cl mice showed markedly elevated PDG mRNA abundance in comparison to NR/NH₄Cl mice, however no differences were detected on protein level (figure 4A, F-G). In parallel, PEPCK mRNA expression was markedly enhanced after an acid-load (2.5-fold) and in both obese groups (figure 5A); O-GI mice given NH₄Cl showed the greatest fold change in mRNA expression (4.4-fold vs 2.2—fold; O-GI and NR groups, respectively). Protein abundance for PEPCK was also increased after an acid load, but similar to PDG, showed no differences among the two acid-loaded cafeteria fed groups (figure 5B-F).

In addition to potential alterations in renal ammoniogenesis, we sought to determine if there were any distal defects in ammonium transport in the kidney, in particular of the Rhesus glycoprotein, Rhcg, in our mice. Rhcg is largely expressed in the connecting tubule and the collecting duct, and plays an important role in apical ammonium extrusion into the final urine [44, 46, 76]. Rhcg mRNA expression was unremarkable between groups, however O-GI mice given an acute acid load showed increased Rhcg mRNA expression levels in comparison to O-GI treated without NH₄Cl treatment (figure 6).

Brush border membrane sodium-proton exchanger activity is reduced in acid loaded obese, glucose-intolerant mice

To further identify mechanisms that could contribute to a reduction in urinary ammonium excretion, we examined the Na⁺/H⁺ exchanger (NHE) activity in the brush border membrane of proximal tubules. Reduced NHE activity was observed in acid-loaded O-GI mice (figure 7).

DISCUSSION

We created a mouse model of diet-induced obesity and glucose-intolerance to examine if changes in the regulation of the glutamine transporter, SNAT3, and enzymes involved in renal ammoniogenesis and gluconeogenesis, PDG and PEPCK could explain the urinary acidification and ammonium excretory defects seen in subjects with UAN and diabetes. After 10 weeks of dietary treatment, cafeteria-diet fed mice fell into two distinct cohorts based on body weight and response to IPGTT. O-IR mice were significantly obese and had severely elevated blood glucose, while NR mice were moderately obese with more normal blood glucose levels. Similar observations have been previously reported in rats [72-74, 77]. Since we used a highly inbred mouse strain (C57/BL6) with very little genetic variance in our study, the underlying reason for this difference remains unknown at present and provides an excellent model to study the impact of obesity related defects on the same genetic and dietary background. We show that challenging these obese, diabetic mice with an oral acid load leads to defective urinary ammonium excretion despite enhanced expression of key ammoniogenic proteins. O-GI mice exhibited strong upregulation of SNAT3, PDG and PEPCK mRNA and protein abundance suggesting enhanced ammoniogenesis. Moreover, the proximal tubular brush border membrane sodium-proton exchanger activity was rather reduced in obese and glucose-intolerant mice whereas Rhcg mRNA expression appeared normal.

Studies by Abate et al. and Sakhaee et al. assessed whether or not insulin resistance is associated with UAN. Most patients with UAN had symptoms of metabolic syndrome and a low urinary pH, but were without changes in urinary ammonium excretion. The ammonium concentration as a major component of net acid excretion, however, was significantly reduced in these subjects [7, 15]. Our results show a slightly higher urinary pH and reduced urinary NH_4^+ excretion in O-GI but not NR mice after an acid challenge, but not at baseline. This may be a species specific response to obesity and/or insulin resistance. However, it should also be noted

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that previous studies reporting decreased urinary pH in patients with metabolic syndrome sampled urine only over 4 to 12 hours [7, 11]. In contrast, our samples were collected over 24 hrs and the differences may thus reflect rather circadian rhythmicity of urine and acid excretion. However, challenging mice with an acute acid load unmasked several defects. O-IR mice showed slightly higher urinary pH, in contrast with clinical data suggesting that patients with UAN and insulin resistance showed consistently low urinary pH values [6-10]. Despite lower NH_4Cl intake in comparison to controls, both groups of obese mice displayed appropriate responses to acid-loading, namely reduced urinary pH and increased NH_4^+ excretion. Importantly, the difference seen between the glucose-tolerant and glucose-intolerant obese cannot be explained by differences in NH_4Cl intake.

The renal manifestations of insulin resistance are not fully understood to date. Bobulescu et al. found that Zucker diabetic fatty rats exhibit reduced urinary ammonium excretion and pH, as well as reduced NHE3 activity in the renal brush border membrane [63]. Interestingly, these rats showed an increase in renal triglyceride content, suggesting a lipotoxic effect of fatty acids in the kidney. In opossum kidney cell lines (OKP), as a model of the PT cell, administration of long-chain fatty acids produced a similar reduction in NHE3 activity, NHE3 membrane surface expression and ammonium excretion. Thus, accumulation of lipids, particularly free fatty acids (FFA) in the kidney may result in alterations in normal, physiological cellular processes, such as ammoniogenesis or gluconeogenesis. The etiological relationship between insulin resistance and low urinary pH has not been identified yet, but a major contribution of the proximal tubule is likely. O-GI mice showed strong upregulation of SNAT3, PDG and PEPCK on both protein and mRNA level, which would imply that ammoniogenesis and likely gluconeogenesis is being maximally fueled by the influx of glutamine into the PT cells.

Ammonium excretion by the proximal tubule involves Na^+/H^+ -exchange activity in the brush border membrane, mainly mediated by the NHE3 isoform. The activity and expression of NHE3 has been linked to ammonium excretion even though in mouse models of NHE3 deficiency no clear defect in ammonium excretion has been reported to date [78]. NHE3 expression and

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activity in vivo and in the OKP cell line is stimulated by insulin [62]. We and Bobulescu et al.

found that NHE3 expression or activity are altered in animal models of obesity and glucose intolerance pointing to a dysregulation of this important transporter [79].

The reduced urinary ammonium excretion may also more distal defects. mRNA expression levels of Rhcg, critical for renal ammonium excretion, however, did not show significant differences between groups. Urinary pH was only slightly more alkaline in O-GI mice and is thus unlikely to account for a reduced driving force for NH_3 secretion.

In summary, a diet-induced mouse model of obesity and glucose-intolerance demonstrates major changes in the regulation of enzymes and transporters involved in proximal tubular ammoniogenesis, gluconeogenesis, and ammonium excretion suggesting that obesity and insulin-resistance lead to a profound dysregulation of this important pathway in renal acid-base and glucose metabolism.

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FIGURE LEGENDS

Table 1

Urine analyses were performed to determine the effect of cafeteria style feeding and NH_4Cl treatment on urinary parameters. Values are means \pm SE of results from analyses for urinary electrolytes, PO_4^{3-} and NH_4^+ relative to creatinine in mice ($n = 8$ mice per group) treated with a standard or cafeteria diet for 10 weeks, with or without NH_4Cl supplementation in the food for the final 2 days. ^a $P \leq 0.05$; ^b $P \leq 0.01$; ^c $P \leq 0.001$, comparisons between control and treated groups. ^d $P \leq 0.05$; ^e $P \leq 0.01$; ^f $P \leq 0.001$, comparisons between cafeteria-fed groups. ^g $P \leq 0.05$; ^h $P \leq 0.01$; ⁱ $P \leq 0.001$, comparisons between acid-loaded groups.

Figure 1

Two tiers of glucose intolerance are unmasked in cafeteria-fed mice after intraperitoneal glucose tolerance testing. Intraperitoneal glucose tolerance tests were performed on all mice ($n = 72$) after 10 weeks of a standard or cafeteria diet. Mice were fasted overnight and blood glucose monitoring was performed every 30 minutes for 2 h after an initial glucose injection (2mg/g body weight), **(A)**. The most severely glucose-intolerant mice were subsequently named Obese, glucose-intolerant (O-GI), while the second tier of mice had moderately elevated blood glucose levels and were called the Non-responders (NR).

Figure 2

Acid challenging cafeteria-fed mice with NH_4Cl reveals a difference in ammonium excretion and urinary acidification in O-GI mice.

Mice were placed in metabolic cages and urine was collected for the final two consecutive 24 h intervals under mineral oil. **(A)** All acid-loaded groups exhibited low urinary pH values in comparison to respective controls, however O-GI mice showed a slightly more alkaline urine

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than NH₄Cl-loaded mice fed a standard diet. **(B)** 24 h urinary ammonium excretion relative to creatinine was significantly reduced in O-GI, but not NR mice. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, $n = 6$ mice/group

Figure 3

O-GI mice show strong upregulation of SNAT3 mRNA and protein abundance after an acid challenge with NH₄Cl.

mRNA expression of SNAT3 was determined in kidneys using semi-quantitative real-time RT PCR in mice fed a standard or cafeteria diet for 10 weeks with or without NH₄Cl supplementation in food, and normalized against HPRT mRNA expression. All acid-loaded groups showed enhanced SNAT3 mRNA expression, with the O-GI group showing the highest upregulation of SNAT3 **(A)**. **(B-K)** Immunoblotting for SNAT3 in total membranes. All blots were stripped and reprobed for β -actin to control for equal loading. Original blots are depicted with bar graphs summarizing normalized data. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ between groups, $n = 5$ mice/ group.

Figure 4

Phosphate-dependent glutaminase is strongly enhanced in O-GI mice after an acid-load.

mRNA expression of PDG was determined using semi-quantitative real-time RT PCR. PDG mRNA was enhanced in all groups after an acid load, and most strongly in the O-GI group given NH₄Cl when compared to acid-loaded control and NR mice **(A)**. **(B-G)** Cytosolic proteins were extracted from whole, non-perfused kidneys and probed for PDG. All blots were stripped and reprobed for β -actin to control for equal loading. Original blots are depicted with bar graphs summarizing normalized data. * $p < 0.05$, ** $p < 0.01$ between groups, $n = 5$ mice/ group.

Figure 5

Phosphoenolpyruvate carboxykinase mRNA expression and protein abundance parallel PDG in cafeteria-fed mice.

RNA was extracted from total kidney homogenates and mRNA expression was determined by semi-quantitative real-time RT PCR. PEPCK mRNA expression was elevated in all acid-loaded groups, however both cafeteria fed groups showed the greatest increase in mRNA expression **(A). (B-F)** Cytosolic proteins were extracted from whole, non-perfused kidneys and probed for PEPCK. All blots were stripped and reprobed for β -actin to control for equal loading. Original blots are depicted with bar graphs summarizing normalized data. * $p < 0.05$, ** $p < 0.01$, *** $p \leq 0.001$ between groups, n = 5 mice/ group.

Figure 6

Normal expression of the ammonia transporter Rhcg

mRNA expression of Rhcg was determined in all groups. * $P \leq 0.05$ between groups.

Figure 7

Sodium/hydrogen exchanger activity is reduced in BBMV from acid-loaded O-GI mice.

BBMV preparations were made and acridine orange quenching experiments were performed **(A).** O-GI mice given an NH_4Cl load had reduced NHE activity relative to respective controls. In contrast, acid-loaded NR mice showed enhanced NHE activity when compared with NR mice without NH_4Cl treatment. * $P \leq 0.05$ and *** $P \leq 0.001$ between groups.

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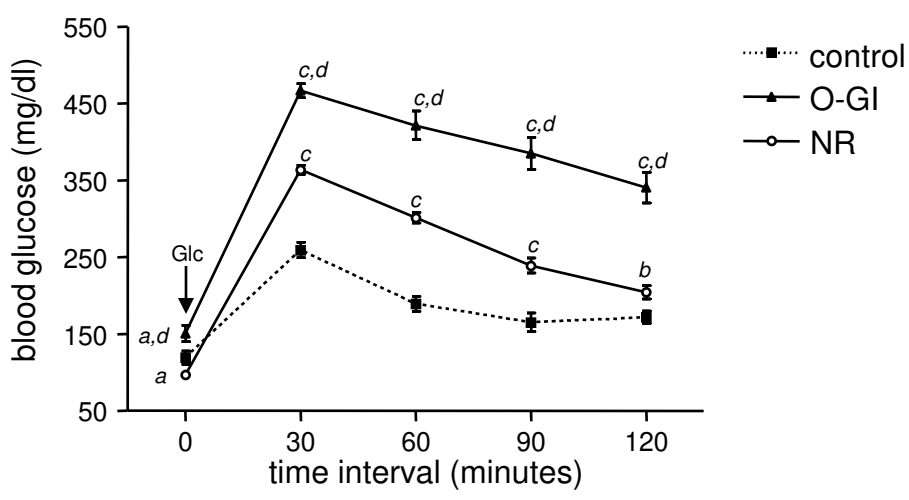
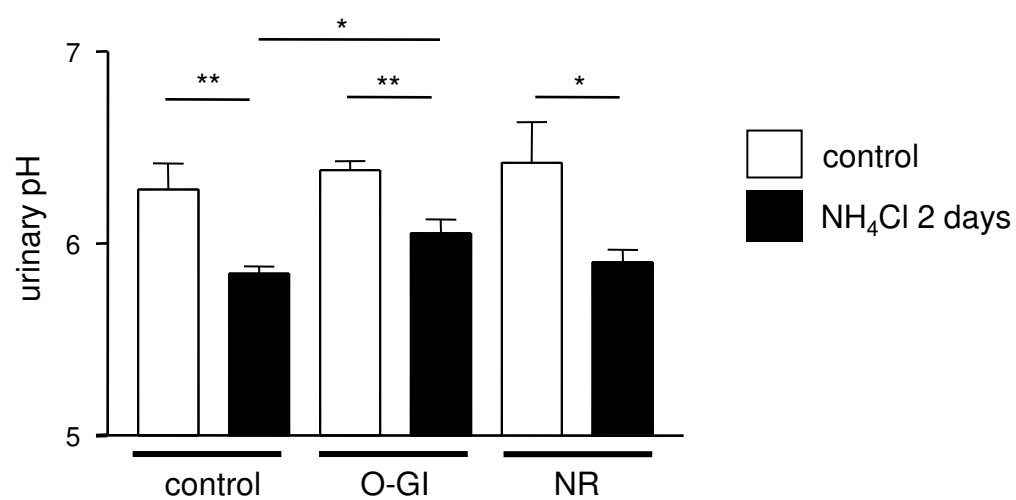


Figure 2

A.



B.

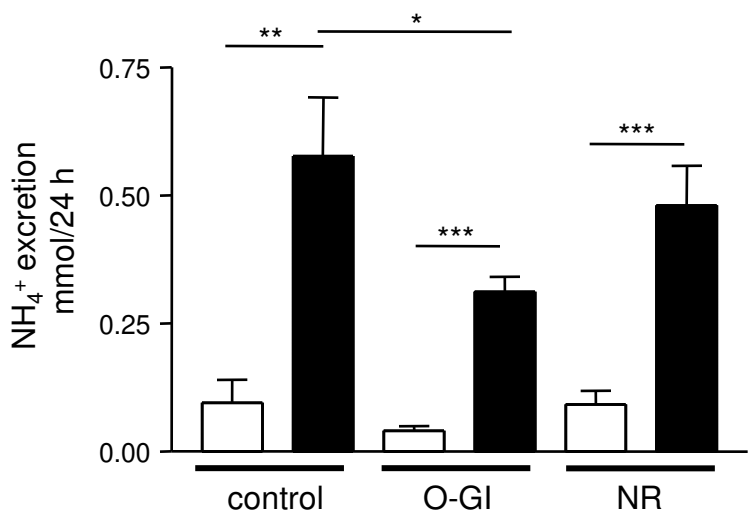
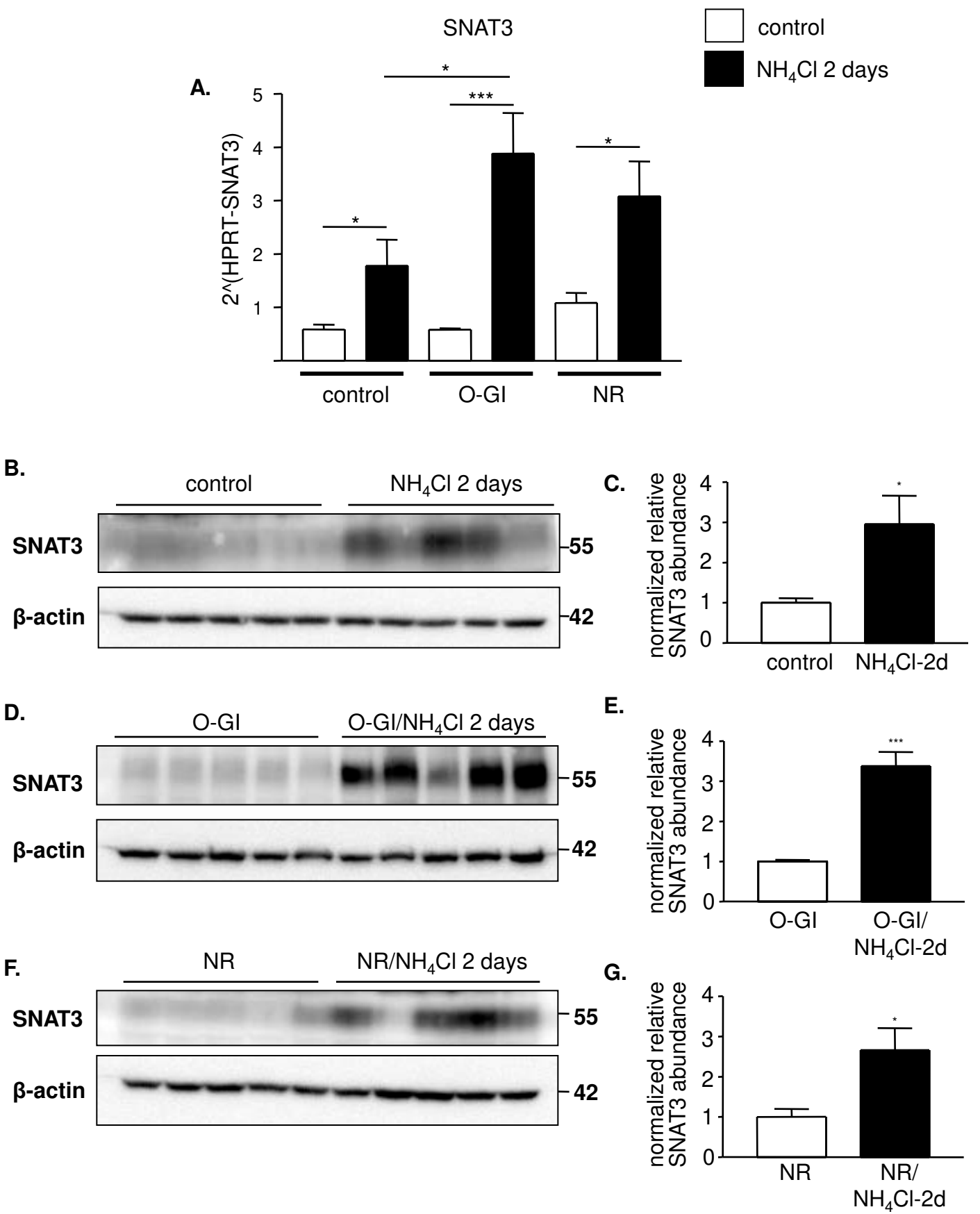


Figure 3



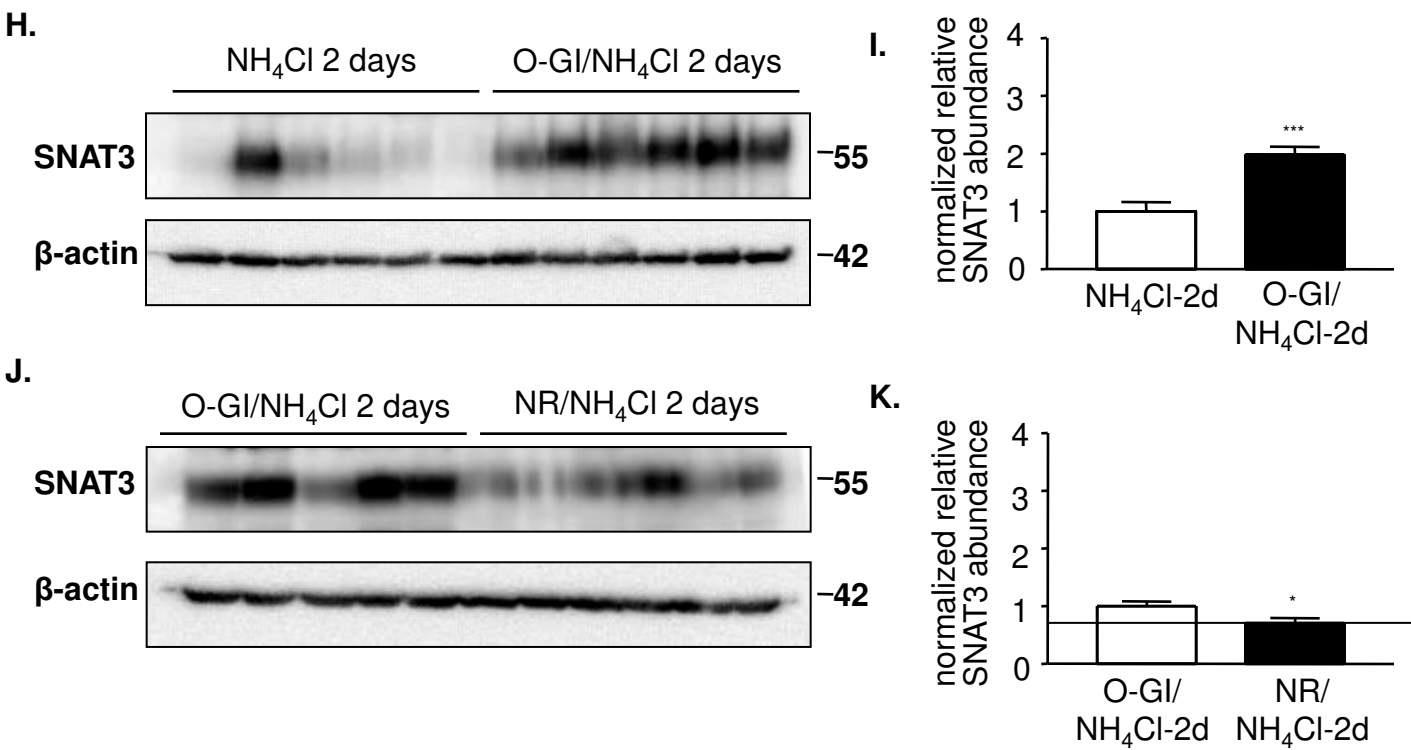


Figure 4

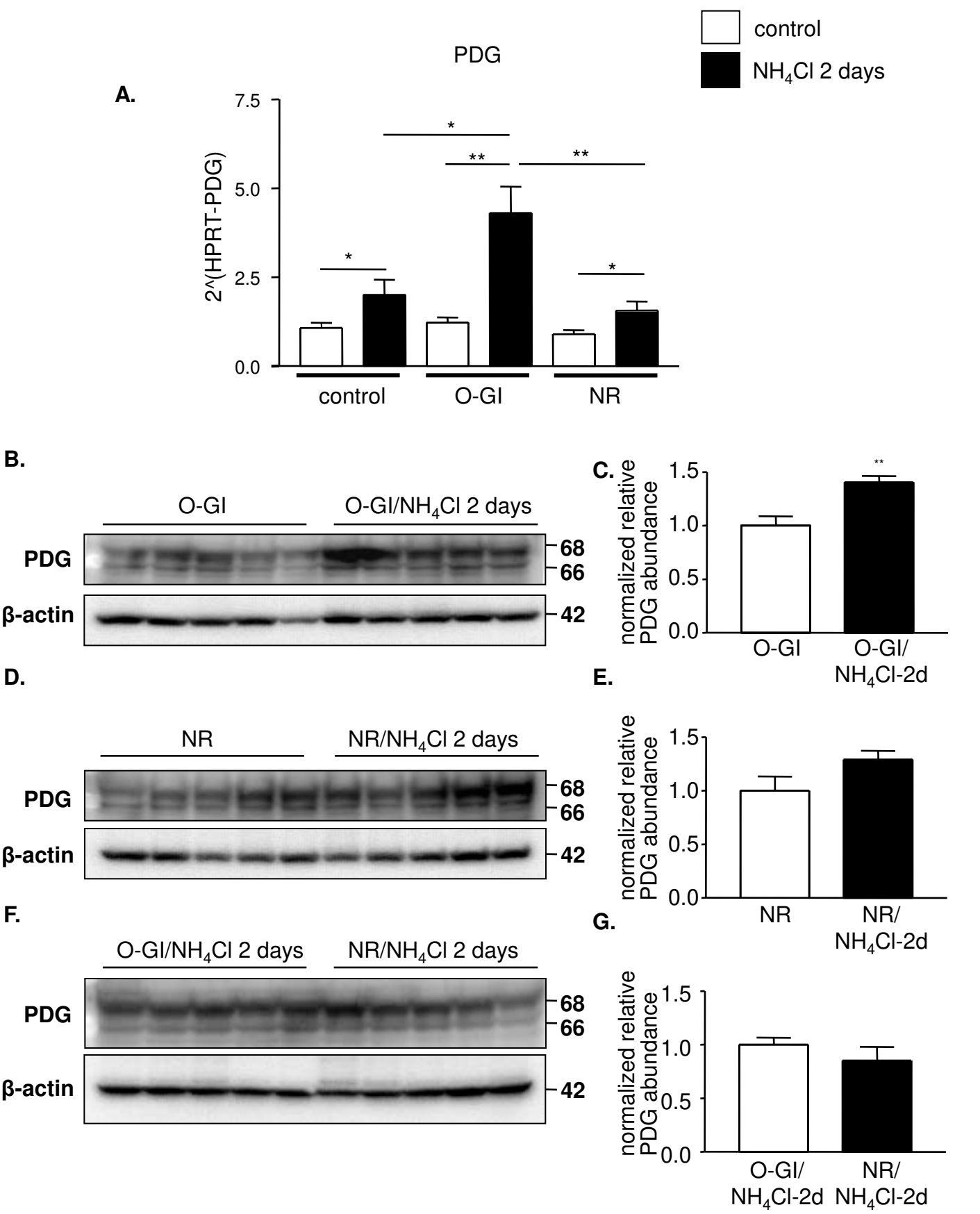


Figure 5

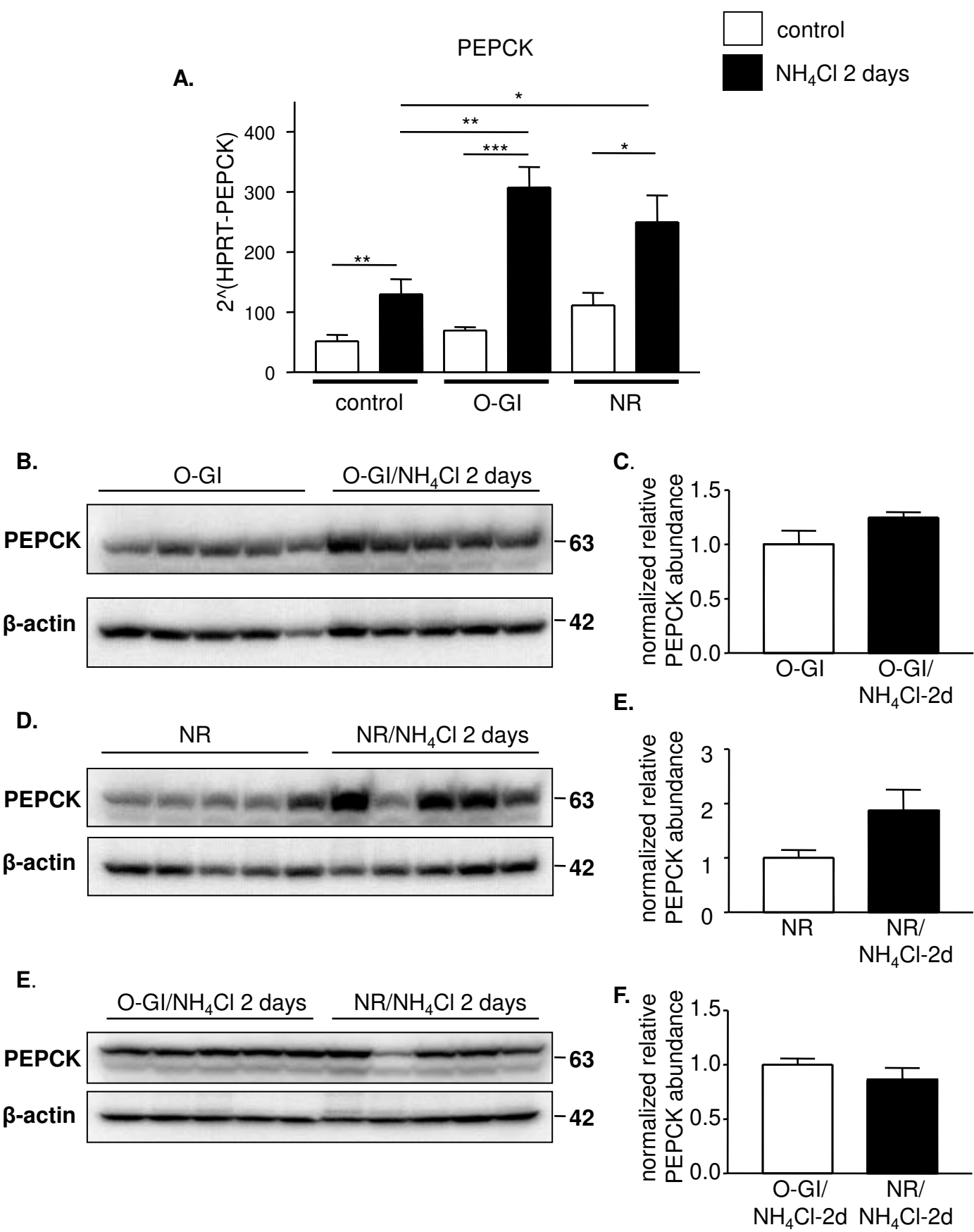


Figure 6

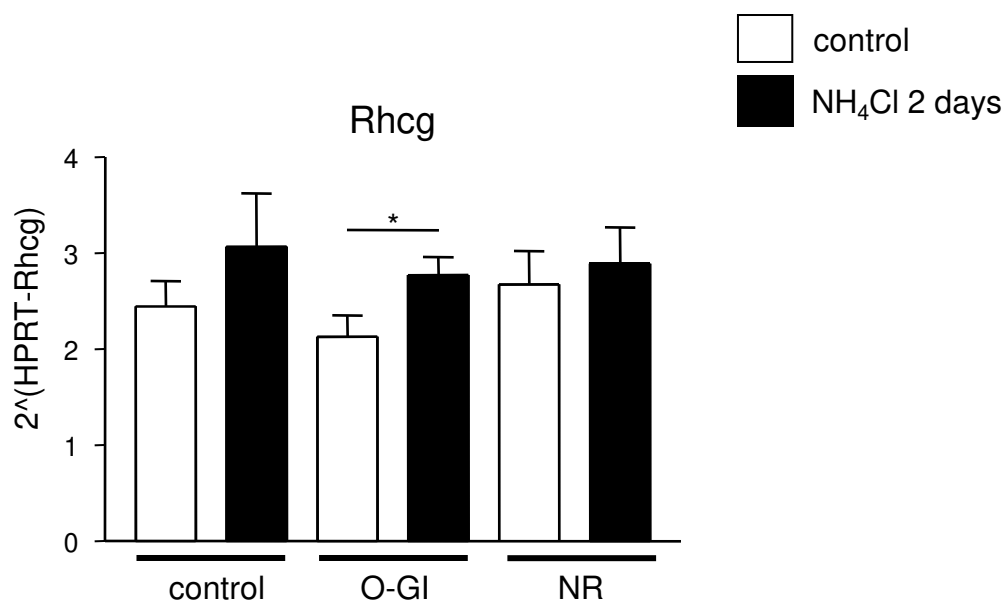


Figure 7

